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Introduction

As cells progress through the various phases of the cell cycle the levels of most of the mammalian cyclins fluctuate dramatically. The major protease involved in the removal of cell cycle proteins is the fully regulated 26S proteasome, a multicatalytic protease responsible for the degradation of polyubiquitinated proteins (Glas et al. 1998). *In vitro*, inhibition of proteasome function prevents cell cycle progression and arrests cells at various stages of the cell cycle. Therefore, proteasome inhibitors like bortezomib (also known as PS-341 or Velcade) or Marizomib (also known as NPI-0052 or salinosporamide A) have been used to target cancer clinically (Kane et al. 2003). However, proteasome inhibitors alone failed to show significant anti-tumor activity and their combination with radiotherapy or established anti-cancer agents seems to be more promising.

Many chemotherapeutic agents as well as radiation have differential efficacy against cancer cells in different phases of the cell cycle. Furthermore, certain chemotherapeutic drugs (Piccinini et al. 2001; Fekete et al. 2005) as well as ionizing radiation (Pajonk et al. 2001) affect proteasome function directly. Exact understanding of when and to which extent proteasome function is affected by a treatment modality is essential in order to better time the application of proteasome inhibitors in the clinic. Investigating the role of the proteasome in different prostate cancer cell subpopulations could be fundamental for the development of more specific and efficient therapies. In fact, both prostate cancer and benign prostatic hyperplasia are believed to arise as a result of changes in the balance between cell proliferation and differentiation (Isaacs et al. 1989; Bonkhoff et al. 1996). Experimental evidence suggests that the malignant transformation originates in a subset of primitive cells meanwhile most cells in an organ do not generate tumors (Huntly et al. 2004; Barker et al. 2009).

In the previous year, we focused on the regulation of the 26S proteasome throughout the cell cycle and on the characterization of low and high proteasome activity prostate cancer cells.

Body

As already mentioned in the previous progress report, the proteasome plays a central role in the progression of the cell cycle and we have hypothesized that it is regulated throughout the different phases of the cell cycle. This year we extended our study by investigating the possible mechanism of this regulation. In order to study the proteasome degradation efficiency in the different cell cycle phases, mimosine, a plant-derived amino acid, was employed. PC-3 cells were treated with the drug for 24hours at a concentration of 400uM and, at different times after the release of the cells from the drug, all three proteasome activities were measured. By adding mimosine to the lysis buffer at the same concentration used in the cell culture, we proved its inability to directly interaction with the 26S proteasome (Fig 1).

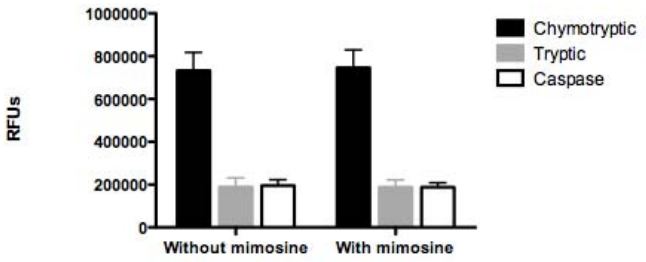


Fig 1. Chymotryptic, tryptic and caspase-like activity in cells lysed in presence/absence of mimosine (400uM).

Our results confirmed the suitability of the drug for the study of the cell cycle.

Cell cycle synchronization was demonstrated not just by FACS analysis of synchronized cells stained with Propidium Iodide, but also by the regulation of cyclins in the different cell cycle phases (Figure 2). In fact, the collapse of various cyclin species, as cells advance from one phase to the next, is due to their rapid degradation. Their fluctuation is tightly coordinated with the schedule of advances through the various cell cycle phases. Cyclins become polyubiquitinated and, as a result, become target of the proteasome. The sole exception to these well-programmed fluctuations is the level of cyclin D, not dramatically varying in the different cell cycle phases.

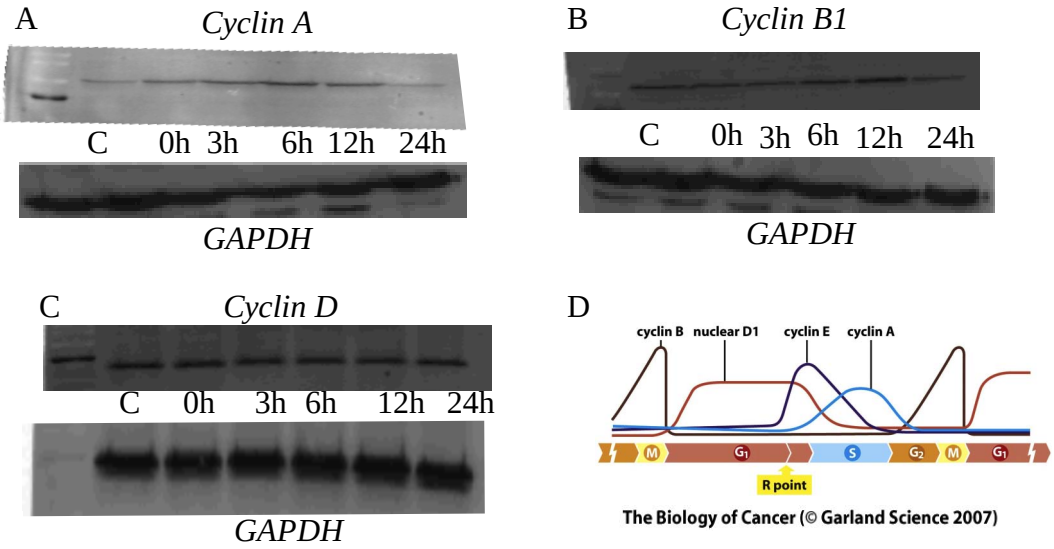
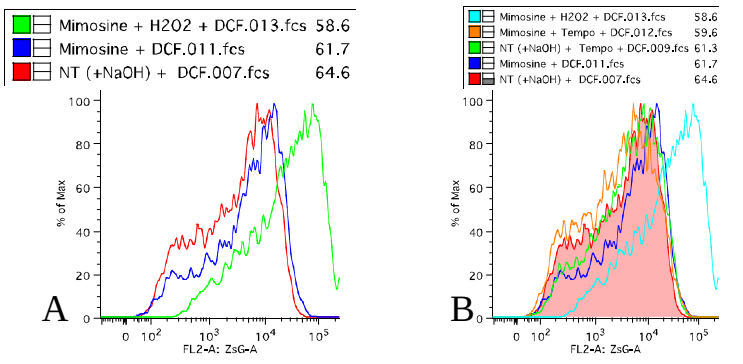
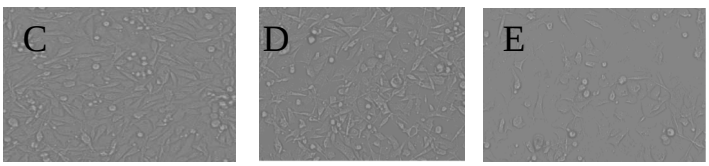


Fig 2. Western blot against Cyclin A (A), B1 (B) and D (C) at different time points from the release of the cells from the drug. GAPDH was used as loading control. D: representative scheme of the regulation of cyclins in the different phases of the cell cycle.

Reactive oxygen species (ROS) can be generated during diverse biological and cellular reactions and they are known to interfere with the cell cycle (Helt et al. 2001). In particular, they are generated synchronously with the normal cell cycle and they have to be controlled at certain level for normal progression of the cell cycle (Takahashi et al. 2004). It has been reported that exogenous ROS arrest the cell cycle at the G2 phase (Savitsky et al. 2002). On the other hand, it has been proposed that treatment with mimosine resulted in production of H_2O_2 (Panopoulos et al. 2005). The mechanism is based on the fact that many ROS-producing enzymes contain heme. Thus, the possibility that mimosine might produce DNA breaks independently of the process of DNA synthesis, but by chelating iron and increasing production of ROS.
Taken these observations into consideration, we tested if the physiologic fluctuation of ROS during the cell cycle was responsible for the drop observed at G1/S boundary and if mimosine treatment affected ROS generation. By using the fluorescent dye 2′,7′-dichlorofluorescein (DCF) in normally cycling and synchronized cells we measured the formation of ROS (Fig 3). A positive control represented by cells treated with H_2O_2 and a sample treated by the superoxide scavenger Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl or 4-hydroxy-tempo) to test if mimosine generated ROS were also added.





mimosine + TEMPO

Fig 3. FACS analysis of ROS upon mimosine treatment. A: level of ROS upon mimosine treatment (blue line) and upon H_2O_2 treatment (green line, positive control). B: level of ROS in presence of the ROS scavenger, TEMPO (orange line). Blue line indicates ROS level upon H_2O_2 treatment (positive control). C, D and E are representative microscopic images of cells normally cycling (A) and treated with mimosine alone (D) or mimosine and TEMPO (E).

Our results show that the level of ROS in synchronized cells is equal to the not synchronized cells, indicating that neither the physiological fluctuation of ROS throughout the cell cycle, nor mimosine treatment is responsible for the drop of proteasome activity. We conclude that the fluctuation of ROS throughout the cell cycle in our case does not represent the mechanism by which the proteasome is regulated throughout the cell cycle. Still seeking a mechanism we hypothesized that a phospho/dephosphorylation of the 26S proteasome complex can be responsible for this fluctuation, however, further investigations are needed.

As described in the previous progress report, we studied the possibility that the reduced efficiency of proteasome activity observed upon irradiation was never more than 50% due to the heterogeneity of the cells in regard to their proteasomes. This year, we have been deeper investigated low and high proteasome activity in two human prostate cancer cell lines. Besides PC-3 cell line, already mentioned in the previous progress report, another prostate cell line, DU145, was stably transduced for the expression of the fluorescent protein, ZsGreen, and the degron from the cODC using a retroviral vector (Vlashi et al. 2009) (Fig 4).

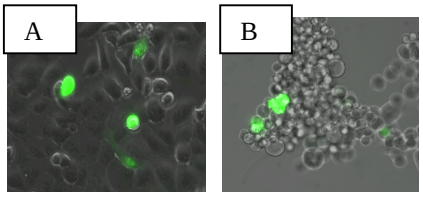


Fig 4. Microscopic images of DU145 cells transduced with ZsG-cODC vector, reporter for proteasome activity, in monolayers (A) and in serum free conditions (B).

In flow cytometry experiments cells were defined as “ZsGreen-cODC-positive” if the fluorescence in the FL-2 channel (FITC) exceeded the one of non-transfected control by at least two orders of magnitude.
In both cell lines, the percentage of ZsGreen positive cells was significantly higher when cells were maintained as prostate spheres compared to monolayer cultures. In PC-3, the percentage of ZsGreen cells in monolayers averaged 0.1% in monolayers and 5% in spheres; in DU145, 0.5% of cells were ZsGreen positive in monolayers compared to 2.17% in spheres.
In both cell lines, treatment with the proteasome inhibitor MG132 caused a significant increase of the green signal, demonstrating the intact expression of the construct ZsGreen-cODC in almost all cells (Figure 5A and B).

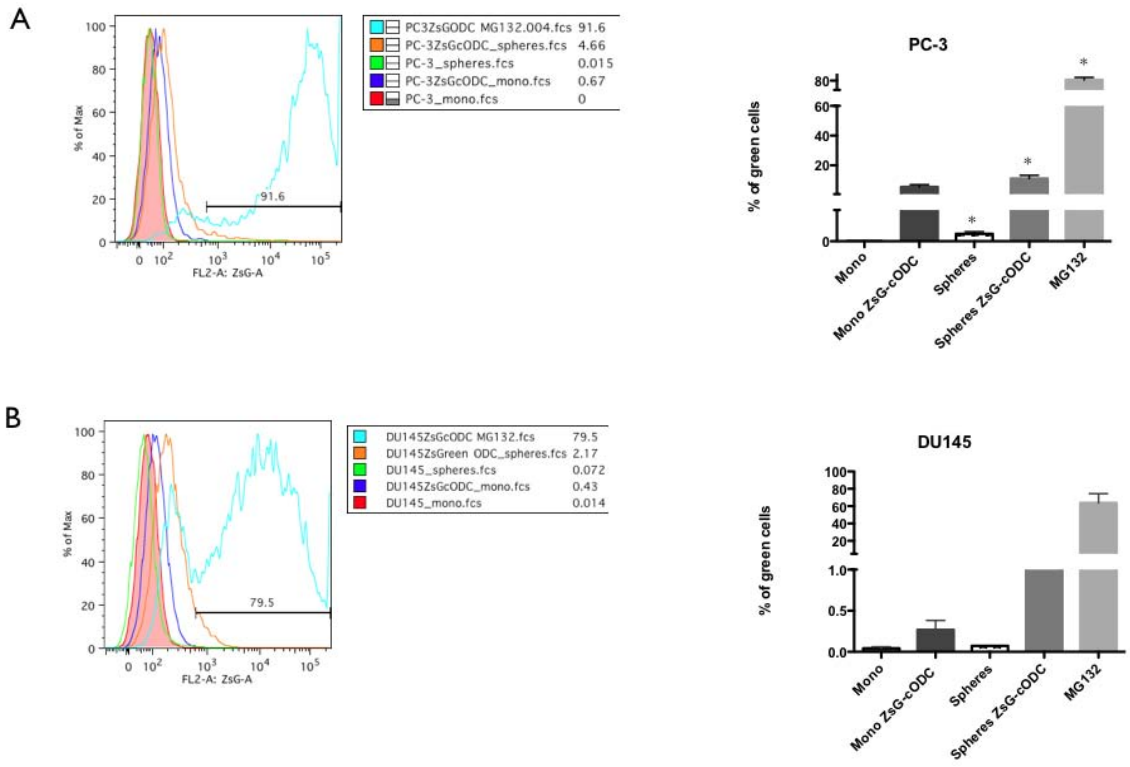


Fig 5. FACS analysis of ZsGreen-cODC expression of PC-3 (A) and DU145 (B) cell lines. Treatment with the proteasome inhibitor MG132 (5μM) overnight was used as a positive control. Column graphs indicate the percentage of ZsGreen negative and positive cells in monolayers versus spheres for both cell lines (n=3; P <0.5). Student's t test was used for statistical analysis.

In order to quantify 26S proteasome function in monolayers and prostate spheres, we performed fluorogenic proteasome function assays measuring chymotryptic, tryptic, and caspase-like activities. Both cell lines showed a significantly reduced tryptic activity when cultured as prostate spheres compared to monolayer cultures. Chymotryptic and caspase-like activities were unchanged (Fig 6).

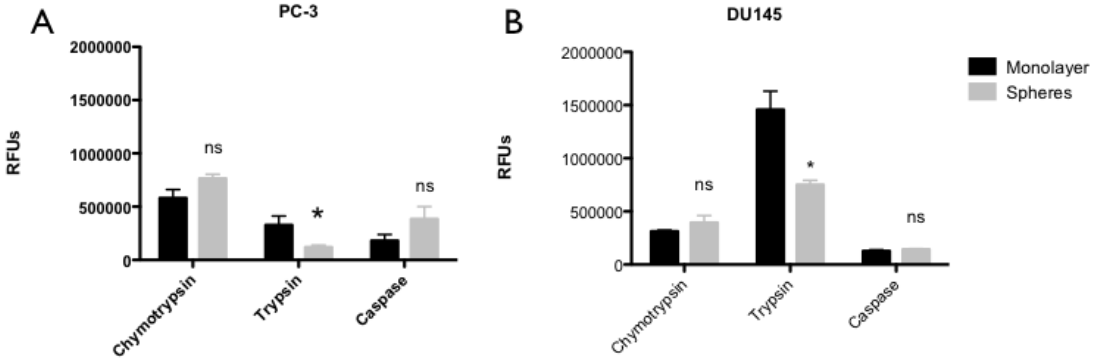
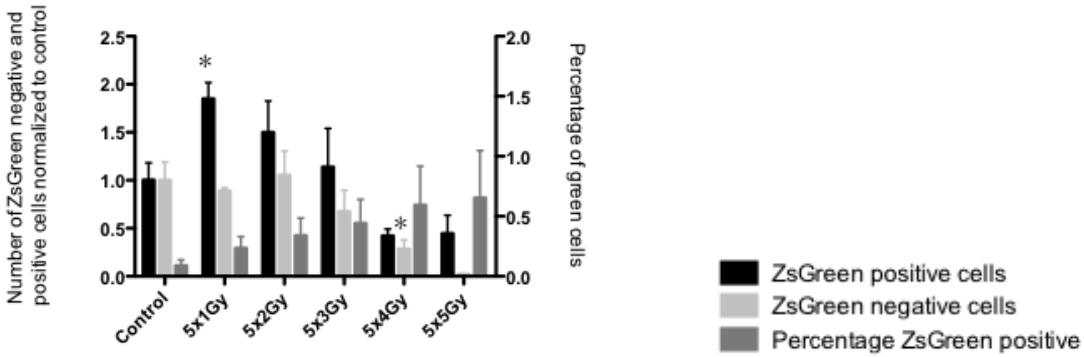


Fig. 6. 26S proteasome activities in PC-3 (A) and DU145 (B). Means± 95% confidence intervals derived from four independent experiments (four replicates per experiment). Student's paired two-tailed test was used.

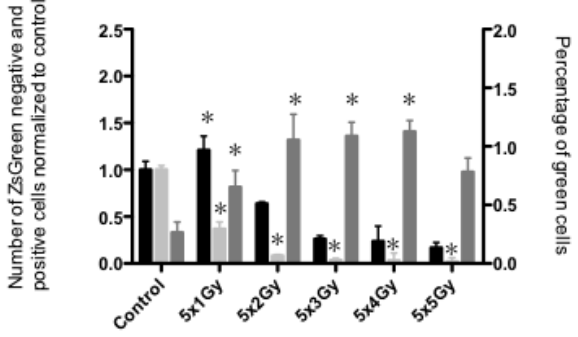
These data suggest the coexistence of two distinct cell populations in prostate cancer with different proteasomal activity. Radiation therapy plays an important role in the cure of prostate cancer. However, the outcome is limited by the existence of a highly radioresistant population. Thus, we wanted to elucidate whether a marked radioresistance may correlate with a particular phenotype. We used the two cell populations, with low (ZsGreen-positive) or high (ZsGreen-negative) proteasome activity cells and exposed monolayers and sphere cultures to different doses of fractionated irradiation (5x1Gy, 5x2Gy, 5x3Gy, 5x4Gy, 5x5Gy). 72h after the last irradiation dose the total number of ZsGreen positive and negative cells and the percentage of positive cells was analyzed by FACS. The number of positive and

negative cells were normalized to un-irradiated controls. For monolayer cultures, the number of cells having low proteasome activity increased significantly upon 5x1Gy irradiation, almost doubling their number (normalized values from 1 to 1.8, n=4, P< .05), while the number of negative cells declined (normalized values from 1 to 0.8) (Fig 7A). Similarly, in spheres the number of cells irradiated with low doses increased (from 1 to 1.2, n=4, P< .05), meanwhile the number of ZsGreen-negative cells dropped with increasing radiation doses (from 1 to 0.2 after 5x5Gy). Remarkably, in both monolayers and spheres the percentage of low proteasome activity cells increased in parallel to the increased radiation dose (Fig 7 B), increasing from 0.1% (un-irradiated) to 0.8% (5x5Gy) (n=4, P < .05) in monolayers, and from 0.3% (un-irradiated) to 1% in spheres (n=4, P < .05).

A



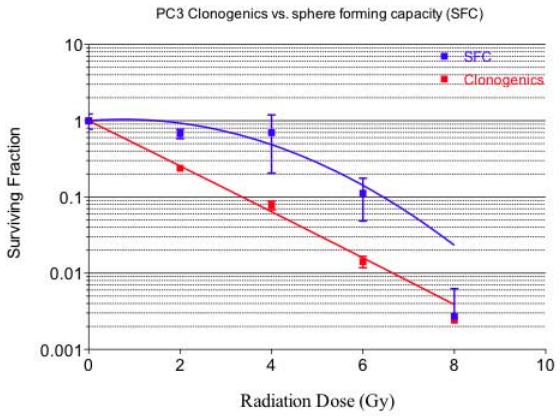
B



ıre 7. Radiation sensitivity of low and easome activity cells. A: number of ZsG itive and positive cells derived from monolayer k and light grey columns, respectively. Results nalized to the total number of living cells. Statis ysis was performed by using the paired, one tc st (P< .05). Dark grey columns (right Y cate the percentage of ZsGreen positive cells irradiation dose. B: number of ZsGreen negc positive cells derived from spheres cultures.

In both cell lines, clonogenic assays performed in monolayer cultures and sphere forming capacity assays were performed to confirm the higher radioresistance of spheres, enriched for cells with low proteasome activity (Fig 8, A and B).

A



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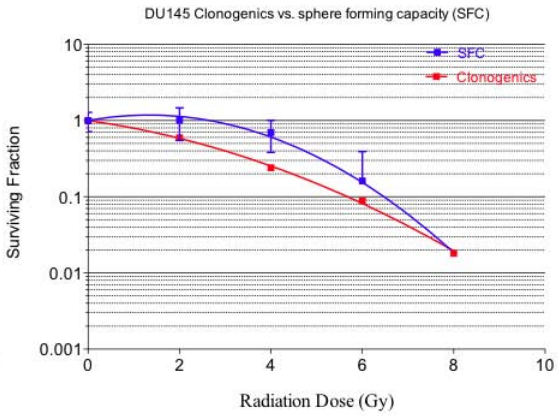


Figure 8. Radiation sensitivity of low and high proteasome activity cells. A: Clonogenic survival of unselected prostate cancer cells (red) and cells enriched for cells wit low proteasome activity (blue)

Studies to determine, which cell type give rise to prostate cancer has been conducted in the past. Although basal and luminal cells have been proposed to represent the cell types of origin for prostate cancer, the heterogeneity of prostate cells and the lack of specific markers limited the outcome (Moscatelli et al. 2010; Richardson et al. 2004; Wang et al. 2009; Goldstein et al. 2010). Basal cells express $CK5^{+}$, $CK8/18^{low}$; luminal cells are devoid of basal cell markers and express $CK8/18^{high}$, but not CK5 (Goldstein et al. 2010); other cells co-express a spectra of basal and luminal CKs (Wang et al. 2001; Kurita et al. 2004). To better characterize the radioresistant population of cells having low proteasome activity, PC-3 cells were stained for different cell surface markers and analyzed by FACS.

Our results indicate that in PC-3, cells with low or high proteasome activity do not differ in the expression of CK8 and CK5. Among the cell population with low proteasome activity, only 20% co-expressed CK8 and CK5, meanwhile the majority (44%) was double negative for the two cytokeratins (Figure 9A). If from one side the low proteasome activity population seems to preferentially express the luminal marker CK8 compared to the basal CK5, on the other side the luminal marker CK18 was expressed in only one third of the low proteasome activity population (Figure 9C). Almost all cells in PC-3 (99% of the whole population) express CD49f (Figure 9B). Only an extremely small percentage of low proteasome activity cells (less than 1%) co-expresses Trop2 and CD49f (Figure 9B) and CD44 was found to be expressed in both, cells with low and high proteasome activity (Figure 9D).

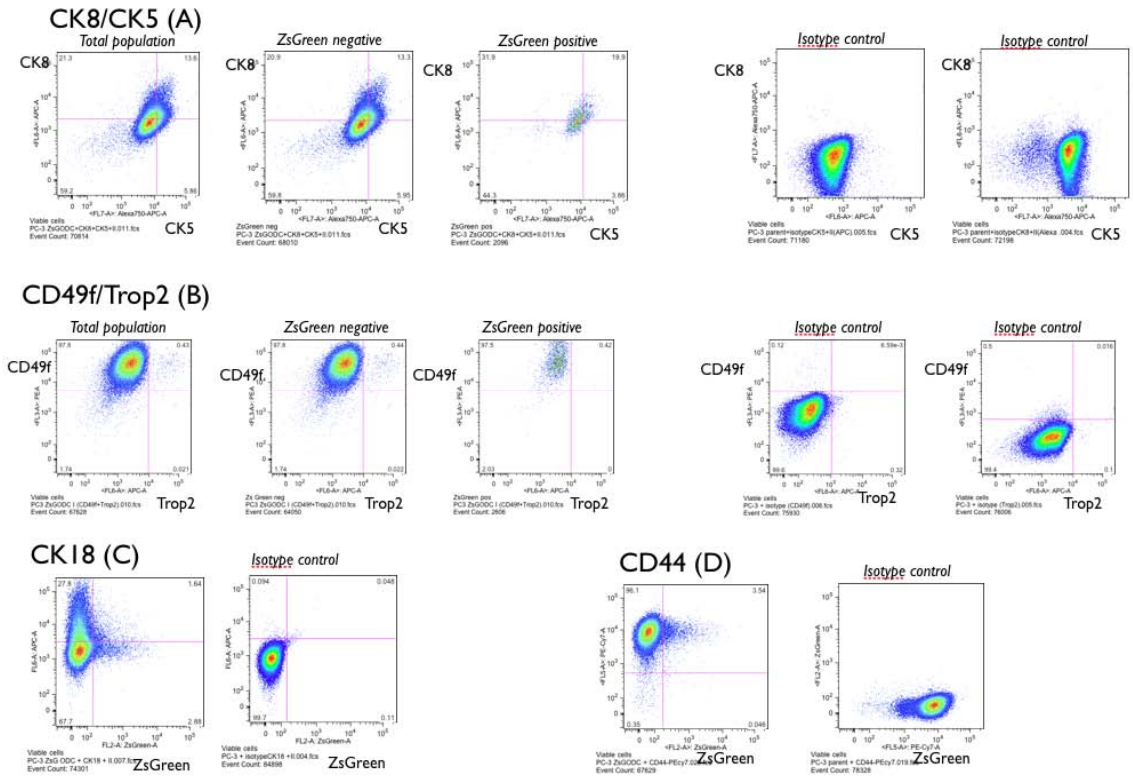


Fig. 9. FACS analysis of PC-3 ZsGreen-cODC cells stained for the expression of CK8/CK5 (A), CD49f/TroP2 (B), CK18 (C) and CD44 (F).

Our findings didn't allow us to assign cells with low or high proteasome activity with a luminal or basal phenotype. In fact, FACS data indicate that the majority of ZsGreen positive cells express the luminal marker CK8, but only a few express another luminal marker, CK18. Interestingly, the observation that only a small percentage of ZsGreen positive cells co-expresses CK8 and CK5, suggests the possibility that the low proteasome activity cells with basal characteristics may give rise to luminal cells or *vice versa*. Furthermore, low proteasome activity cells are CD49f positive and Trop2 negative, overlapping again with a luminal and basal phenotype at the same time.

Next, we wanted to extend our investigations elucidating if proteasome activity is linked to self-renewal and tumorigenicity. Sphere forming capacity assays were performed by growing PC-3ZsGreen-cODC cells as monolayers and sorting them into ZsGreen-negative or -positive cells at a density of 1 cell/well into 96-well plates. After 3 weeks,

the number of spheres formed per well was then counted and expressed as a percentage of the initial number of cells plated. Cells were also plated into serum-free media into 100 mm suspension dishes at 10,000 cells/ml, and allowed to form prostatespheres for 15 days. These cells were used for secondary sphere forming experiments. For both primary and secondary generation, three independent experiments were performed.

ZsGreen positive cells had statistically significant higher sphere forming capacity compared to ZsGreen negative. For PC-3 15% spheres were formed from the ZsGreen positive cells and 5% ZsGreen negative (P=0.04). For DU145 8% spheres were formed from the ZsGreen positive population and 4% from the negative (P=0.02) (Figure 10).

The secondary sphere formation assays performed in PC-3 cell line showed a higher secondary sphere forming capacity in cells with low proteasome activity compared to the ZsGreen negative population, with the ZsGreen positive cells redistributing in positive and negative. Similar results were obtained in DU145 cell line (Figure 10). These results indicate how cells with low proteasome activity are characterized by a significant higher self-renewal capacity compared to the non-green cells.

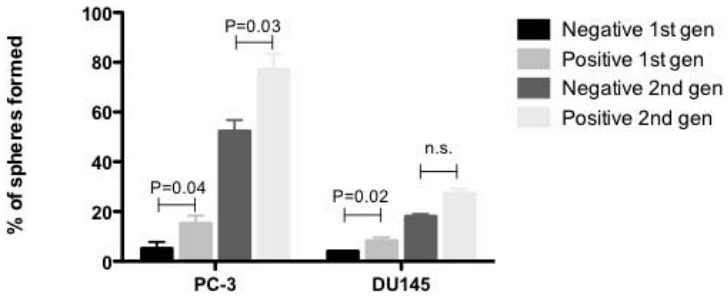


Figure 10. Sphere forming capacity assay. Number of spheres formed from the ZsGreen-negative and -positive cell population after sorting at one cell per well into 96-well plates (first and second generation). Second generation of spheres was obtained by maintaining ZsGreen negative and positive cells sorted cells in serum free media and re-plating them in 96-well plates. Means± S.E.Ms from four independent experiments.

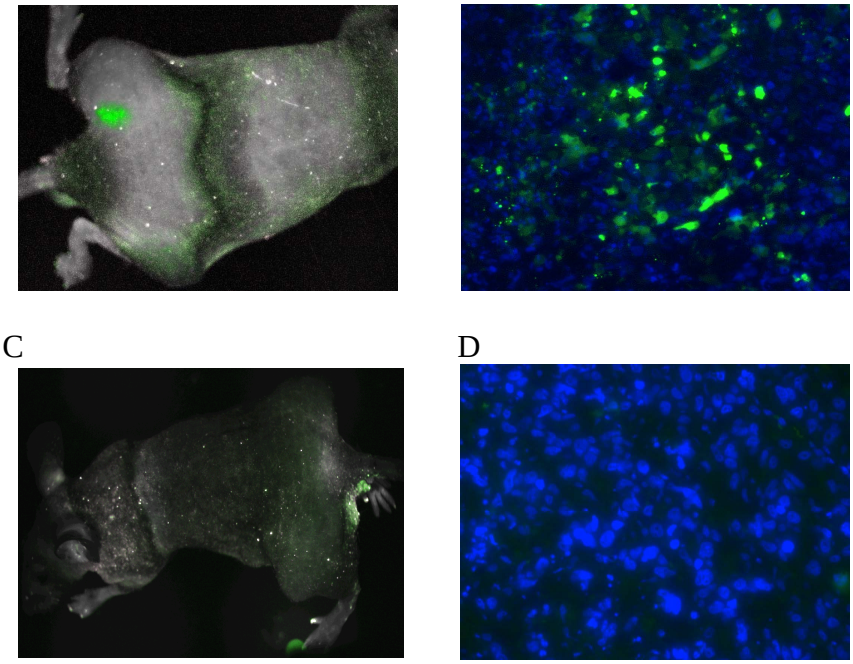
Tumorigenicity was addressed in an *in vivo* study. PC-3ZsGreen-cODC cells were sorted by FACS in ZsGreen-negative and -positive cells and injected subcutaneously into both thighs of Nu/Nu 6-8 weeks old male mice. 10^6, 10^5, 10^4, 10^3, 10^2, 10 negative cells and 10^6, 10^5, 10^4, 10^3,10^2 and 10 positive cells were injected per inoculum. Tumor growth was monitored on a weekly basis and mice were sacrificed when tumor size reached the protocol guidelines requiring euthanasia. Mice injected with ZsGreen-negative and -positive cells were imaged for the presence of ZsGreen positive cells with the Maestro In Vivo Imaging System (UCLA) before being sacrificed. The injection of 10 cells, whether ZsGreen negative or positive, didn't give rise to any tumor. At 100 cells per inocculum, only ZsGreen positive cells were able to generate tumors (Table 1) and the difference in TD50 was 1 log.

Table 1*. Tumor formation (number of tumors and percentage) in Nu/Nu male mice injected with ZsGreen negative and positive sorted cells from 10 to 1,000,000 cells.*

Negative		Positive	
# of cells	# of tumors	# of cells	# of tumors
10	0/10	10	0/10
100	0/12	100	3/12 (25%)
1,000	3/8 (37.5%)	1,000	8/10 (80%)
10,000	3/10 (30%)	10,000	6/12 (50%)
100,000	9/10 (90%)	100,000	4/4 (75%)
1,000,000	4/4 (100%)	1,000,000	-----

If tumors formed, there was no difference in size between tumors formed upon the injection of ZsGreen-negative and -positive cells (data not shown). To track the injected cells, mice were monitored by *in vivo* imaging: tumors derived from ZsGreen positive cells gave rise to tumors exhibiting fluorescence signal (Figure 11, A and B). Tumors derived from ZsGreen negative cells gave rise to tumors not exhibiting any fluorescence signal (Figure 11, C and D).

A B



C D

Figure 11. Tumorigenicity of ZsGreen-negative and-positive cells. A. Tumor from Nu/Nu mouse injeceted with 100 ZsGreen positive cells. B Section from the same tumor deparaffinized and stained with DAPI. C. Tumor from Nu/Nu mouse injected with 1,000,000 ZsGreen negative cells. D. B Section from the same tumor deparaffinized and stained with DAPI.

Key Research Accomplishment:

1. Establishment of a prostate cancer cell line (DU145) transfected with a tagged Rpn11 subunit and a ZsGreen-cODC construct to investigate proteasome heterogeneity in prostate cancer cells.
2. Identification of two subpopulations of cells in prostate cancer cells.
3. Characterization of cells with low and high proteasome activity in prostate cancer using luminal and basal markers
4. Characterization on radiosensitivity of cell with low and high proteasome activity in prostate cancer.
5. Characterization of cells with low and high proteasome activity in prostate cancer using luminal
6. and basal markers
7. Characterization of tumorogenicity and self-renewal capacity of cells with low and high proteasome activit

Reportable outcomes:

Poster: Lorenza Della Donna, Chann Lagadec, Erina Vlashi, Carmen Dekmezian, Puneet Souda, Julian Whitelegge, and Frank Pajonk. Regulation of 26S proteasome activity by radiation during the different phases of the cell cycle.

56th annual Meeting of the Radiation Research Society, Grand Wailea Resort Hotel and Spa, Maui, September 25-29.

Poster: Lorenza Della Donna, Chann Lagadec, Erina Vlashi, Carmen Dekmezian, Julian Whitelegge, Puneet Souda and Frank Pajonk. The Activity of the 26S Proteasome is Modulated during the Different Phases of the Cell Cycle. 1st PPDUP (Proteomics of Protein Degradation & Ubiquitin Pathways) Conference, June 6th-8th, 2010, Vancouver, British Columbia, Canada (*Excellent Poster Presentation Award*).

Manuscript: Lorenza Della Donna*, Chann Lagadec, Erina Vlashi, Carmen Dekmezian, and Frank Pajonk. Radioresistance of prostate cancer cells correlates with low proteasome activity (*submitted*).

Conclusion:
We conclude that cells in prostate cancer are heterogeneous and that a radioresistant cell population in prostate cancer exists that is associated with low 26S proteasome activity. In the present study we reported on their characterization based on radiation sensitivity, tumorigenicity and expression of protein surface markers.

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